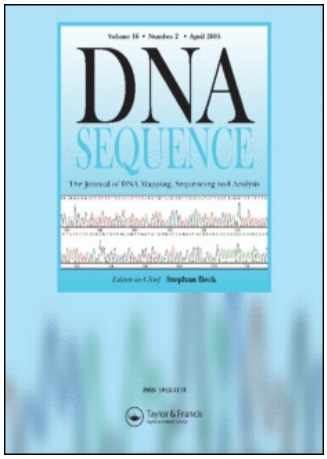

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Analysis of rILERS , an Isoleucyl-tRNA Synthetase Gene Associated with Mupirocin Production by Pseudomonas fluorescens NCIMB 10586

Vidhya Rangaswamy [a]; Gustavo Hernández-Guzmán [a]; Kevin A. Shufran [b]; Carol L. Bender [a]
[a] Department of Entomology and Plant Pathology, 127 Noble Research Center, Oklahoma State University, Stillwater, OK 74078, USA.
[b] USDA-ARS, Plant Science and Water Conservation Research Laboratory, Stillwater, OK 74074, USA.

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Analysis of *rILERS*, an Isoleucyl-tRNA Synthetase Gene Associated with Mupirocin Production by *Pseudomonas fluorescens* NCIMB 10586

VIDHYA RANGASWAMY[a,*], GUSTAVO HERNÁNDEZ-GUZMÁN[a], KEVIN A. SHUFRAN[b] and CAROL L. BENDER[a]

[a]*Department of Entomology and Plant Pathology, 127 Noble Research Center, Oklahoma State University, Stillwater, OK 74078, USA;* [b]*USDA-ARS, Plant Science and Water Conservation Research Laboratory, Stillwater, OK 74074, USA*

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Some strains of *Pseudomonas fluorescens* produce the antibiotic mupirocin, which functions as a competitive inhibitor of isoleucyl-tRNA synthetase (ILERS). Mupirocin-producing strains of *P. fluorescens* must overcome the inhibitory effects of the antibiotic to avoid self-suicide. However, it is not clear how *P. fluorescens* protects itself from the toxic effects of mupirocin. In this report, we describe a second gene encoding isoleucyl-tRNA synthetase (*rILERS*) in *P. fluorescens* that is associated with the mupirocin biosynthetic gene cluster. Random mutagenesis of the mupirocin-producing strain, *P. fluorescens* 10586, resulted in a mupirocin-defective mutant disrupted in a region with similarity to ILERS, the target site for mupirocin. The *ILERS* gene described in the present study was sequenced and shown to be encoded by a 3093 bp ORF, which is 264 bp larger than the *ILERS* gene previously identified in *P. fluorescens* 10586. *rILERS* from *P. fluorescens* is most closely related to prokaryotic or eukaryotic sources of ILERS that are resistant to mupirocin. Interestingly, the relatedness between *rILERS* and the *ILERS* previously described in *P. fluorescens* 10586 was low (24% similarity), which indicates that *P. fluorescens* contains two isoforms of isoleucyl-tRNA synthetase.

Keywords: Isoleucyl-tRNA synthetase; Plasposon; Polyketide; Pseudomonic acid

Database Accession No.: AY079084

INTRODUCTION

Mupirocin (pseudomonic acid) is an antibiotic produced by some strains of the gram-negative, aerobic bacterium *Pseudomonas fluorescens*. Isotopic labeling studies with [^{13}C]-acetate previously established that mupirocin originates from the polyketide pathway (Feline *et al.*, 1977), and this hypothesis was supported by the isolation of mupirocin-defective mutants disrupted in polyketide synthase genes (Rangaswamy and Bender, unpublished). Mupirocin is a competitive inhibitor of isoleucyl-tRNA synthetase (ILERS) and functions by preventing the incorporation of isoleucine into newly synthesized proteins (Hughes *et al.*, 1980). The depletion of isoleucine-charged tRNA within cells results in the rapid arrest of protein synthesis. Yanagisawa *et al.* (1994) speculated that mupirocin is a bifunctional inhibitor that interacts with ILERS as an analog of both isoleucine and ATP.

Mupirocin exhibits a high level of antibacterial activity against staphylococci, streptococci, *Haemophilus influenzae* and *Neisseria gonorrheae* but is less active against gram-negative bacilli and anaerobes (Sutherland *et al.*, 1985). Mupirocin has been registered for use in over 90 countries for

*Corresponding author. Address: Industrial Biotechnology, Reliance Life Sciences, C/o RPL, SSO, Annexe 4, Jamnagar, Gujarat 361140, India. Tel.: +91-0288-291986. E-mail: vidhya_rangaswamy@ril.com

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the treatment of skin infections and is one of the most successful topical antibiotics for the elimination of *Staphylococcus aureus*(Cookson, 1998). With the increasing use of mupirocin, resistance among staphylococci has emerged (Bradley *et al.*, 1995). High-level resistance (MIC $>500\,\mu g\,ml^{-1}$) is generally associated with a plasmid-borne gene designated *mupA*, which encodes a mupirocin-resistant form of isoleucyl-tRNA synthetase (Hodgson *et al.*, 1994). Low-level resistance to mupirocin (MIC $<100\,\mu g\,ml^{-1}$) is more common, can be selected *in vitro* with increasing concentrations of mupirocin, and is thought to arise from point mutations within the chromosomally-encoded staphylococcal *ILERS* (Cookson, 1998; Antonio *et al.*, 2002).

Strains of *P. fluorescens* that produce mupirocin must overcome the inhibitory effects of the antibiotic to avoid self-suicide. Hughes *et al.* (1980) proposed that mupirocin-producing strains of *P. fluorescens* produce an altered form of ILERS that is insensitive to mupirocin. In support of this hypothesis, Isaki *et al.* (1990) cloned the ILERS gene from *P. fluorescens* 10586 on a recombinant plasmid named pBROC128, and *Escherichia coli* transformants containing pBROC128 showed elevated resistance to mupirocin. The *ILERS* gene from plasmid pBROC128 was subsequently sequenced and overproduced in *E. coli* (Yanagisawa *et al.*, 1994). However, mutagenesis of the region flanking *ILERS* in *P. fluorescens* 10586 had no effect on mupirocin production, which suggests that the *ILERS* gene does not map with the mupirocin biosynthetic gene cluster (Whatling *et al.*, 1995).

In this report, we describe a gene encoding a second isoform of isoleucyl-tRNA synthetase (*rILERS*) from *P. fluorescens* NCIMB 10586. This gene maps to a cosmid clone containing polyketide synthase genes, which is consistent with the biosynthetic origin of mupirocin (Feline *et al.*, 1977). A transposon insertion in *rILERS* rendered *P. fluorescens* 10586 incapable of mupirocin synthesis, which suggests that mupirocin production is dependent on a functional copy of *rILERS*, presumably to avoid self-toxicity.

MATERIALS AND METHODS

Bacterial Strains and Media

The bacterial strains and plasmids used in this study are listed in Table I. *P. fluorescens* 10586 was obtained from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, Scotland). *P. fluorescens* and derivatives were maintained on King's medium B (KMB) (King *et al.*, 1954) or mannitol-glutamate (MG) medium (Keane *et al.*, 1970) at 28°C. *Escherichia coli* DH5α and *Bacillus subtilis* were grown in Terrific Broth (TB) or Luria-Bertani (LB) medium at 37°C (Sambrook *et al.*, 1989). Antibiotics were added to media at the following concentrations ($\mu g\,ml^{-1}$): ampicillin, 100; kanamycin, 25 (*E. coli*) or 50 (*P. fluorescens*), and tetracycline, 25.

Plasposon Mutagenesis

The plasposon pTnModOKm (Dennis and Zylstra, 1998) was mobilized into *P. fluorescens* via triparental matings using the helper plasmid pRK2013. Briefly, the recipient *P. fluorescens* cells, the donor *E. coli* containing pTnModOKm, and the helper *E. coli* HB101(pRK2013) were grown overnight on solid media. Cells were resuspended in 1 ml H_2O, and equal volumes of the suspensions were combined, collected on a 0.2 μm filter, and incubated on KMB agar overnight at 28°C. Cells were then removed from the filter and suspended in 10% glycerol. Aliquots (50 μl) of the suspension were plated on MG medium containing kanamycin at $50\,\mu g\,ml^{-1}$ to select for plasposon mutants.

Bioassay for Mupirocin

P. fluorescens colonies were cultured on mupirocin production agar (MPA) overnight at 28°C (Whatling *et al.*, 1995). Plates were overlaid with a mixture of the indicator strain *B. subtilis* (5 ml, grown to late log phase) and 5 ml of LB soft agar medium containing 50 μl of 5% (w/v) 2,3,5 triphenyltetrazolium chloride (TTC). After incubation at 37°C for 6–8 h, mupirocin

TABLE I Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Escherichia coli DH5α	$\Delta(lacZYA - argF)_{U169}$	Sambrook *et al.*, 1989
Pseudomonas fluorescens		
NCIMB 10586	Mup^+	NCIMB
28.1	Km^r Mup^- mutant with plasposon insertion in *rILERS*	This study
Plasmids		
pTnModOKm	Km^r, plasposon used for mutagenesis	Dennis and Zylstra, 1998
pRK7813	Tc^r, broad host range cosmid vector	Jones and Gutterson, 1987
pRK2013	Km^r, helper plasmid used in triparental matings	Figurski and Helinski, 1979
p16.40	Tc^r, cosmid clone containing putative PKS and mupirocin resistance genes	This study

Abbreviations: NCIMB, National Collection of Industrial, Food and Marine Bacteria; Mup, mupirocin.

production was evident by the inhibition of *B. subtilis* (clear zones surrounded by a red hue due to the reduction of TTC). When assayed using this procedure, wild-type *P. fluorescens* produced an inhibition zone of ~2–3 cm in diameter.

DNA Manipulations

Mini- and large-scale plasmid isolations were performed with the Wizard kit (Promega, Madison, WI) or Qiagen midi-preparation kit (Chatsworth, CA). Selected clones were mobilized from *E. coli* to *P. fluorescens* by triparental matings using the helper plasmid pRK2013. Plasmid DNA was isolated from *P. fluorescens* as described previously (Kado and Liu, 1981). Colony hybridization techniques were carried out by standard methods (Sambrook *et al.*, 1989). For radioactive probes, α-^{32}P(dCTP) was purchased from ICN Biomedicals (www.icnbiomed.com). DNA probes were radiolabeled using the RadPrime DNA labeling kit as recommended by the manufacturer (Gibco-BRL, Gaithersburg, MD).

A genomic library of *P. fluorescens* 10586 was constructed in the cosmid vector pRK7813. Genomic DNA was isolated from *P. fluorescens* by standard procedures (Sambrook *et al.*, 1989), partially digested with *Sau*3AI, and 40–50 kb fragments were ligated into the *Bam* HI site of pRK7813. The ligation mixture was packaged using the Gigapack III XL packaging kit from Stratagene (La Jolla, CA). A total of 1700 transformants were isolated and maintained as glycerol stocks at −70°C.

DNA Sequence Analysis

Automated DNA sequencing was performed with Ampli*Taq* DNA polymerase, an ABI 373A apparatus and the ABI PRISM primer cycle sequencing kit (Perkin–Elmer, Foster City, CA). Automated DNA sequencing and oligonucleotide synthesis were provided by the Oklahoma State University Recombinant DNA/Protein Resource facility. A series of subclones was generated in pBluescript SK + (Stratagene, La Jolla, CA) and sequenced with the T3 and T7 primers. Gaps were filled by generating sequence directly from cosmid p16.40 using internal primers. Entire sequence was generated from both strands and sequence data were aligned and homology searches were executed with LaserGene's MegAlign for Windows v. 3.15 (http://www.dnastar.com/) and the BLAST package of the National Center for Biological Information (http://www.ncbi.nlm.nih.gov).

Phylogenetic Analysis

Nucleotide data (3909 total characters) was aligned using the ClustalV method in MegAlign (LaserGene version 5.0). A phylogenetic tree was constructed by maximum parsimony analysis using PAUP version 4.0 (Swofford, 2002). Bootstrap analysis was performed with 1000 replicates and a heuristic search. Gaps were treated as missing and all characters were weighted equally.

Nucleotide Sequence Accession Number

The nucleotide sequence of *rILERS* and flanking DNA was deposited in GenBank as accession number AY079084.

RESULTS AND DISCUSSION

Identification of the rILERS Gene by Plasposon Mutagenesis

Random mutagenesis of *P. fluorescens* 10586 was carried out using the plasposon pTnModOKm. Thirty-three mutants from a total of 1700 were defective in mupirocin production when analyzed by the bioassay described above. The plasposon and flanking DNA were excised from each mutant with *Bam* HI, religated, and transformed into *E. coli* DH5α. DNA flanking the insertion site was sequenced using primers designed from pTnModOKm (GenBank accession #AF061921). Using this

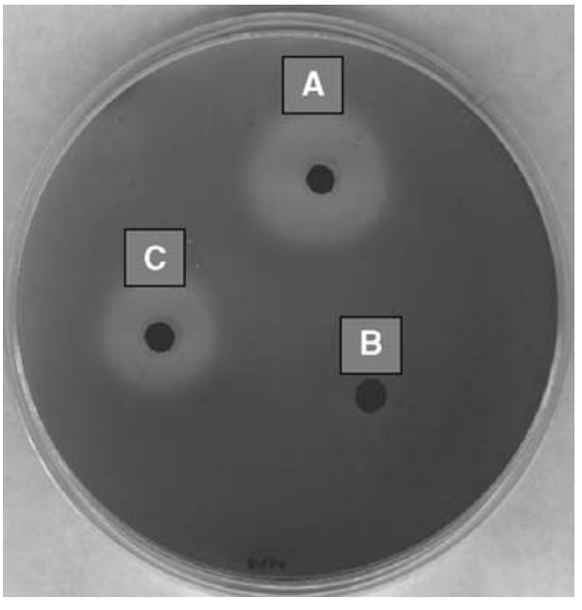

FIGURE 1 Bioassay for mupirocin production. *P. fluorescens* colonies were inoculated to mupirocin production agar, incubated as described in "Materials and Methods" section, and then overlaid with *Bacillus subtilis* and TTC. Mupirocin production was visualized by the inhibition of *B. subtilis* (cleared areas surrounding the *P. fluorescens* colonies). **A**, Zone of inhibition produced by the mupirocin-producing wild-type strain, *P. fluorescens* NCIMB 10586; **B**, lack of inhibition with the mupirocin-defective mutant 28.1; and **C**, inhibition zone restored to mutant 28.1 containing the cosmid clone p16.40.

approach, the plasposon insertion site was localized in 31 mupirocin-defective mutants (Rangaswamy and Bender, unpublished results). Mutant 28.1 was chosen for further study because sequence analysis indicated that the plasposon had inserted into a region with similarity to ILERS, the target site for mupirocin.

Mupirocin production was restored to mutant 28.1 by cosmid p16.40 from the genomic library of *P. fluorescens* (Fig. 1). Cosmid p16.40 also complemented a number of mupirocin-defective mutants that contained insertions in regions encoding polyketide synthase genes (Rangaswamy and Bender, unpublished). These results indicate that the *ILERS* gene disrupted in mutant 28.1 maps adjacent to the biosynthetic loci encoding mupirocin. The association of antibiotic resistance genes with biosynthetic loci is a common self-protection mechanism and has been demonstrated for many antibiotic gene clusters including bacitracin in *Bacillus licheniformis*, vancomycin in *Enterococcus faecalis*, and daunorubicin in *Streptomyces peucetius* (Guilfoile and Hutchinson, 1991; Evers and Courvalin, 1996; Neumuller *et al.*, 2001).

Sequence Analysis of rILERS

The nucleotide sequence of the *ILERS* gene identified in the present study (designated *rILERS*) is shown in Fig. 2. The *rILERS* gene is encoded by a 3093 bp ORF,

```
   1 agtcctggtgataggggggcggcaagacaccatggtgtcgttccaggactcactgagcct
  61 gggagagattctgcccaatgcgcaggtgcgcatcatggacgattgggggcactacacgct
 121 tttctctgatacacaagagagtgtatccaaggttatgctggggtttctgcaaacgctgga
 181 agcgtttgactgtcgtaccgtgccacctgcttttctcacccctttttgtgcgccgtcagt
 241 tgtatcggccgccagtcgacagggtgaggcagacgtggattgacgggcgatggcggcggc
 301 agaccgccaggtaaggtaaagccccgaggggtgctttttttgtttttactgacaggtgt
 361 gactg atg agtacggaaggaagtgggccggttagatttccggcaatggaagatgcggtac
         M  S  T  E  G  S  G  P  V  R  F  P  A  M  E  D  A  V    18
 421 tcgagcggtgggaaaaagaaaagacgttcgagcaatccatcagcgcccgtgagggtaagc
     L  E  R  W  E  K  E  K  T  F  E  Q  S  I  S  A  R  E  G  K    38
 481 cggtgtacgtattttatgacggcccgccgtttgctaccggcctgccgcactacggccata
     P  V  Y  V  F  Y  D  G  P  P  F  A  T  G  L  P  H  Y  G  H    58
 541 ttctgacttcctatatcaaagacgtcataccgcgttaccagacgatgctcggcaaacagg
     I  L  T  S  Y  I  K  D  V  I  P  R  Y  Q  T  M  L  G  K  Q    78
 601 tcccacgccgctggggctgggattgccacggcttgccggtggagttcgaagtcgagaagg
     V  P  R  R  W  G  W  D  C  H  G  L  P  V  E  F  E  V  E  K    98
 661 ccatgggcttcaagtccaagcgcgatattctcgagtttggcgtggagcagttcaacgacg
     A  M  G  F  K  S  K  R  D  I  L  E  F  G  V  E  Q  F  N  D   118
 721 agtgcagagagctggtgctcaagtacgccgatgactggcgtggctttgtcaaccggatgg
     E  C  R  E  L  V  L  K  Y  A  D  D  W  R  G  F  V  N  R  M   138
 781 gccgttgggtcgatttcgatggcgcctacaagaccatggataacgactacatggagtcgg
     G  R  W  V  D  F  D  G  A  Y  K  T  M  D  N  D  Y  M  E  S   158
 841 tgctgtggggctttaaaaccttgcatgacaaggggcatgtctacgagcgcggcaagatcg
     V  L  W  G  F  K  T  L  H  D  K  G  H  V  Y  E  R  G  K  I   178
 901 tgccttactgcgtgcgttgccagacggtgttgtcgaatttcgaggcgcgcctggacgacg
     V  P  Y  C  V  R  C  Q  T  V  L  S  N  F  E  A  R  L  D  D   198
 961 ccttccgcccgcgccgcgatatgtccgcctatgtcaagttcaggcaacaagaccgcccgg
     A  F  R  P  R  R  D  M  S  A  Y  V  K  F  R  Q  Q  D  R  P   218
1021 acactttcttcctggcatggaccaccacaccctggacgttgcctgccaacgtcgcactgg
     D  T  F  F  L  A  W  T  T  T  P  W  T  L  P  A  N  V  A  L   238
1081 ccgtggccgccgatgaaaactatgtgtgcatcgagcacggcgaagagcgcctatggctgg
     A  V  A  A  D  E  N  Y  V  C  I  E  H  G  E  E  R  L  W  L   258
1141 ccgaaggttgcctgggcggcttgttcgatgagccggtgatcctggaacgctgtaccggcg
     A  E  G  C  L  G  G  L  F  D  E  P  V  I  L  E  R  C  T  G   278
1201 cagagctggctgggctgcgttatctgccggtggtcggcgaggtgatcgatgcctcggccc
     A  E  L  A  G  L  R  Y  L  P  V  V  G  E  V  I  D  A  S  A   298
1261 atcgcgtggtcaccgccgacttcgtacagatgggcgatggctctggcattgtccacattg
     H  R  V  V  T  A  D  F  V  Q  M  G  D  G  S  G  I  V  H  I   318
1321 cccctgcgttcggtgaggacgacgccttgctcgggcagcaatacgagttgcctgcaccta
     A  P  A  F  G  E  D  D  A  L  L  G  Q  Q  Y  E  L  P  A  P   338
1381 accctgttcgcgacgacggtacctttccgatgcggtggcgcagtatgccgggcagaata
     N  P  V  R  D  D  G  T  F  S  D  A  V  A  Q  Y  A  G  Q  N   358
1441 ttttcgaggccacgccgcgcatccttgcagatctgaagagcagtggcttgctgttcaagc
     I  F  E  A  T  P  R  I  L  A  D  L  K  S  S  G  L  L  F  K   378
1501 aagaacagatcgaacacaactatccacactgctggcgttgcgataaccctctgatctatc
     Q  E  Q  I  E  H  N  Y  P  H  C  W  R  C  D  N  P  L  I  Y   398
1561 gcgcggtagagtcctggttcatccgcgcgtcggcgctgcgcgagcagttggtggaaaaca
     R  A  V  E  S  W  F  I  R  A  S  A  L  R  E  Q  L  V  E  N   418
1621 acagccaggtcaactgggtgcccgagcatgtgaaggaagggcgcttcggggactggatcc
     N  S  Q  V  N  W  V  P  E  H  V  K  E  G  R  F  G  D  W  I   438
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```
1681 gtaatgcccgcgattgggcggtgtcacgcaaccgtttctggggtgcgcccatcccggtat
     R  N  A  R  D  W  A  V  S  R  N  R  F  W  G  A  P  I  P  V   458
1741 ggcgctgtgaccagtgcggcaccgtcgaggtgatgggcagcatcgcgcagatcgaagcgc
     W  R  C  D  Q  C  G  T  V  E  V  M  G  S  I  A  Q  I  E  A   478
1801 gttccgggcgcaaggtcgaagacctgcatgtgcctcatatcgacgagcatcgtttcgcct
     R  S  G  R  K  V  E  D  L  H  V  P  H  I  D  E  H  R  F  A   498
1861 gccagtgctgcgagggcaccatgagtcgggtgaccggtgtcttcgattgctggttcgaat
     C  Q  C  C  E  G  T  M  S  R  V  T  G  V  F  D  C  W  F  E   518
1921 cgggcgcaatgccgttcgccagtcggcactacccgttcgaaaacaagcaggagttcgaac
     S  G  A  M  P  F  A  S  R  H  Y  P  F  E  N  K  Q  E  F  E   538
1981 agactttccctgccgacttcatcgtcgagtaccttgcgcagacccgcggttggttctaca
     Q  T  F  P  A  D  F  I  V  E  Y  L  A  Q  T  R  G  W  F  Y   558
2041 cgatgatggtcatctccaccggctgtttcgagcagaacccccttcaagaacgccatgtgcc
     T  M  M  V  I  S  T  G  C  F  E  Q  N  P  F  K  N  A  M  C   578
2101 acggggtgattctggccaaggacggtcgcaagatgtccaagcgcctgaagaactacccca
     H  G  V  I  L  A  K  D  G  R  K  M  S  K  R  L  K  N  Y  P   598
2161 acccgatggatctcatgcagacccacggttcggacgccttgcgcgtggccttgctggcat
     N  P  M  D  L  M  Q  T  H  G  S  D  A  L  R  V  A  L  L  A   618
2221 cgccggtctgcaagggagaggacatcaagttcagtgaagagtcggtgcgcgacgtggtgc
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2341 accagttcagtccttccggcgaccttggccagcccctggacaatgtcctggaccactact
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2401 tgttgcatgagttggcggcgctggaatcggatatcaagatgtggatggagtctctggatt
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     P  S  I  D  R  Q  Y  L  S  Y  E  L  A  D  E  M  S  S  L  R   798
2761 acttgatcgccagcgtgcgcaatgtgcgcgaaaccaatggggtttcgcagaagtttccgt
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2941 agcccgtggtggtattgatcttctccttgctcggcaagcgactgggcccggcgatgaagg
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3001 cggtcaccacagcggtgaaggctggagagtatgtaatcgatgaacaggggggctggttg
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3061 ccgcagggcagacgatccagccccacgagttcgagcgtcgcctgaccgtgcgtgacacgc
     A  A  G  Q  T  I  Q  P  H  E  F  E  R  R  L  T  V  R  D  T   918
3121 tcaataacgtcgggattgtcgagaacatggtggtctggctggacttggacatcgatgcct
     L  N  N  V  G  I  V  E  N  M  V  V  W  L  D  L  D  I  D  A   938
3181 cgctcaagcgcgaaggcgccgtacgtgagctcaaccgcaggctgcaagacctgcgcaaga
     S  L  K  R  E  G  A  V  R  E  L  N  R  R  L  Q  D  L  R  K   958
3241 aagccaagctgggctacaccgaaaaagtcgacatcgccgtgctcggcggtgcctatgtcg
     K  A  K  L  G  Y  T  E  K  V  D  I  A  V  L  G  G  A  Y  V   978
3301 atgagatcctggtgcaccacgaggactggctcaagagccagttactggtccagagcttgt
     D  E  I  L  V  H  H  E  D  W  L  K  S  Q  L  L  V  Q  S  L   998
3361 tgcgcagcgatcttgaggcgccgctggcagtggacgaagtcgagctgcccgagggcgacc
     L  R  S  D  L  E  A  P  L  A  V  D  E  V  E  L  P  E  G  D  1018
3421 ctgtgcgtattcaactgcgccgtagcgtactggcctgaggcaatgccgacctggtgccgc
     P  V  R  I  Q  L  R  R  S  V  L  A  *                      1031
```

FIGURE 2 Nucleotide and deduced amino acid sequence of *rILERS* from *P. fluorescens* 10586. The translational start is shown in **boldface**, and the translational stop is indicated in **bold** and denoted with an asterisk (*). Amino acids associated with the Rossman nucleotide-binding fold are shaded; residues implicated in ILERS activity are shaded and underscored; and motifs resembling a Zn-binding site are indicated in **bold** and underscored.

which is 264 bp larger than the *ILERS* gene previously identified in *P. fluorescens* 10586 (Yanagisawa *et al.*, 1994). A potential ribosome-binding site is present 8–12 bp upstream of the *rILERS* translational start. The *rILERS* gene product contains motifs similar to the HIGH and KMSKS signature sequences, which are characteristic of the class I amino acyl-tRNA synthetases (Brown and Doolittle, 1995; Nureki *et al.*, 1998). In rILERS, these appear as HYGH (residues 55–58) and KMSKR (residues 589–593) (Fig. 2).

The rILERS sequence contains the invariable histidine and glycine residues but deviates in one of two variable amino acids from the HIGH consensus. A tyrosine residue at position 56 replaces the isoleucine residue found in majority of the eubacterial enzymes. This substitution is also found in *Caenorhabditis elegans*, *Homo sapiens* and *Mycobacterium tuberculosis* ILERS.

The HIGH and KMSKS signature sequences are indicative of the Rossman nucleotide-binding fold, which is conserved in class I aminoacyl-tRNA synthetases (Fig. 3) and is the active site for ATP binding (Ribas de Pouplana and Schimmel, 2001). In a recent study, ILERS was sequenced from 31 *S. aureus* strains with varying degrees of mupirocin sensitivity. In strains where the MIC for mupirocin was 8–256 $\mu g\,ml^{-1}$, point mutations were identified near the consensus sequence KMSKR (Antonio *et al.*, 2002).

Two other consensus sequences appear in rILERS that have been implicated in isoleucyl-tRNA synthetase activity. WCISR (corresponding to WAVSR, residues 444–448 in rILERS) has been implicated in the activation of isoleucine (Schmidt and Schimmel, 1995). Furthermore, mutational and structural studies with the consensus sequence GWD (amino acids 84–86 in *P. fluorescens* rILERS) demonstrated that these residues bind to the amino moiety of isoleucine via the aspartate residue (Nureki *et al.*, 1998).

Further analysis indicated the presence of a putative metal-binding domain in rILERS. In *E. coli*, ILERS was shown to bind two zinc ions per monomer (Xu *et al.*, 1994). One zinc is bound within the N-terminal domain of ILERS, and the second zinc-binding site is located in the C-terminus of the enzyme (Landro *et al.*, 1994). Although it is not known whether rILERS binds Zn, its primary sequence contains motifs resembling the Zn-binding domains in ILERS from *E. coli* and *M. thermoautotrophicum* (Jenal *et al.*, 1991). The elements C^{182}-V-R-C and C^{389}-W-R-C of *P. fluorescens* rILERS constitute a potential Zn-binding domain (Fig. 2), and the spatial separation between the cysteine pairs is similar to ILERS from *M. thermoautotrophicum* (Jenal *et al.*, 1991).

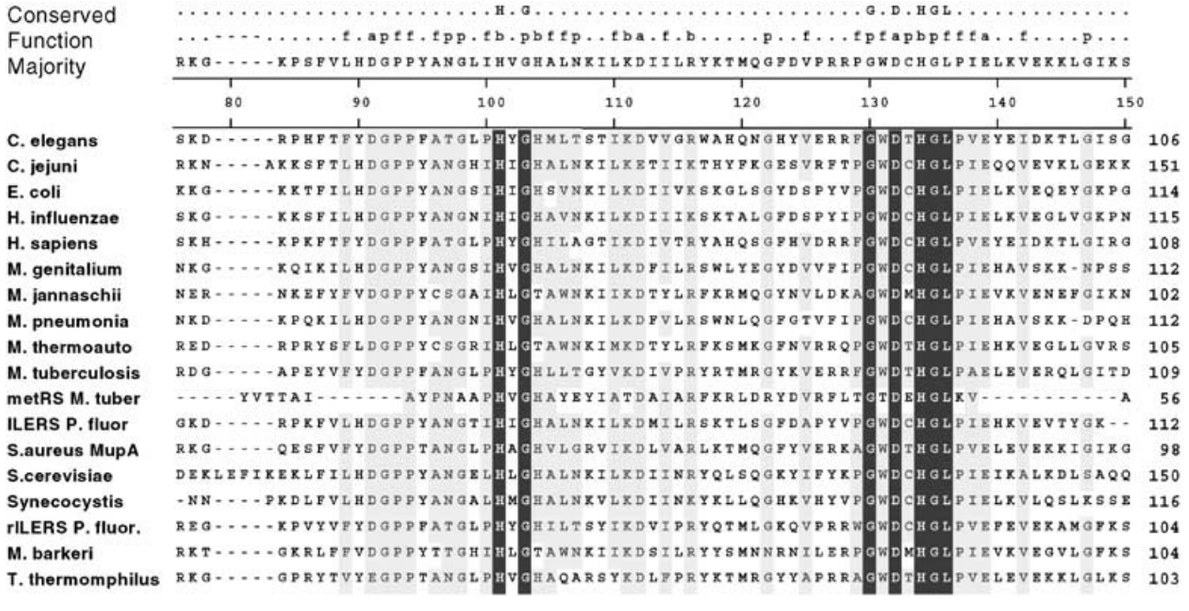

FIGURE 3 Multiple sequence alignment of selected isoleucyl-tRNA synthetases (ILERS) and methionyl-tRNA synthetase. Accession numbers are from the SWISS-PROT or GenBank databases and are shown in parentheses. **C. elegans**, *Caenorhabditis elegans* ILERS (Q21926); **C. jejuni**, *Campylobacter jejuni* ILERS (P41257); **E. coli**, *Escherichia coli* ILERS (P00956); **H. influenzae**, *Haemophilus influenzae* ILERS (P43824); **H. sapiens**, *Homo sapiens* ILERS (P41252); **M. genitalium**, *Mycoplasma genitalium* ILERS (B64238); **M. jannaschii**, *Methanococcus jannaschii* ILERS (Q58357); **M. pneumoniae**, *Mycoplasma pneumoniae* ILERS (P75258); **M. thermoauto**, *Methanobacterium thermautotrophicum* ILERS (O27428); **M. tuberculosis**, *Mycobacterium tuberculosis* ILERS (Q10765); ***metRS* M. tuber**, methionyl-tRNA synthetase from *M. tuberculosis* (O05593); **ILERS P. fluor**, *Pseudomonas fluorescens ILERS* (P18330); **S. aureus MupA**, mupirocin-resistant form of ILERS from *S. aureus* (P41368); **S. cerevisiae**, mitochondrial ILERS from *Saccharomyces cerevisiae* (P48526); **Synechocystis**, *Synechocystis* sp. ILERS (P73505); *rILERS* **P. fluor**, *P. fluorescens* ILERS associated with the mupirocin biosynthetic gene cluster (this study; AY079084); **M. barkeri**; *Methanosarcina barkeri* ILERS (AAF65673.1); and **T. thermophilus**; *Thermus thermophilus* (P56690).The ruler represents an alignment over 76 amino acids in the *S. cervesiae* mitochondrial ILERS. Conserved residues ("**conserved**"), functionally similar residues ("**function**"), and the consensus sequence ("**majority**") are indicated above the ruler. Dashes (–) are incorporated to maximize the alignments. Residues conserved in all proteins are highlighted in blue; residues conserved in the majority of the proteins are highlighted in yellow. Residues associated with the Rossman nucleotide-binding fold (101–104) and ILERS activity (130–132) are indicated.

Comparison of *P. fluorescens rILERS* with other Isoleucyl-tRNA Synthetases

Multiple sequence alignments of *P. fluorescens* rILERS revealed significant relatedness to ILERS from *M. tuberculosis* (58% similarity), *Homo sapiens* (57% similarity), *Methanobacterium thermoautotrophicum* (52% similarity), *Caenorhabditis elegans* (56% similarity), and MupA from *S. aureus* (56% similarity). These relationships are reflected in the phylogenetic tree constructed using the multiple sequence alignments (Fig. 4). *rILERS* from *P. fluorescens* is most closely related to prokaryotic or eukaryotic sources of ILERS that are resistant to mupirocin (Brown *et al.*, 1998; Sassanfar *et al.*, 1996).

Interestingly, the relatedness between *rILERS* and the ILERS previously described in *P. fluorescens* 10586 was low (26% identity, 24% similarity), which indicates that *P. fluorescens* contains two isoforms of isoleucyl-tRNA synthetase. Similar observations were made for *S. aureus*, which contains two forms of ILERS, one that is mupirocin-sensitive and a second form that is mupirocin-resistant (MupA) (Gilbart *et al.*, 1993). The *mupA* gene, which confers high-level mupirocin resistance (MIC > 256 $\mu g\,ml^{-1}$) is plasmid-borne (Gilbart *et al.*, 1993) and highly divergent (34% amino acid identity) from the ILERS in mupirocin-sensitive *S. aureus* strains (Hodgson *et al.*, 1994). As noted above, rILERS

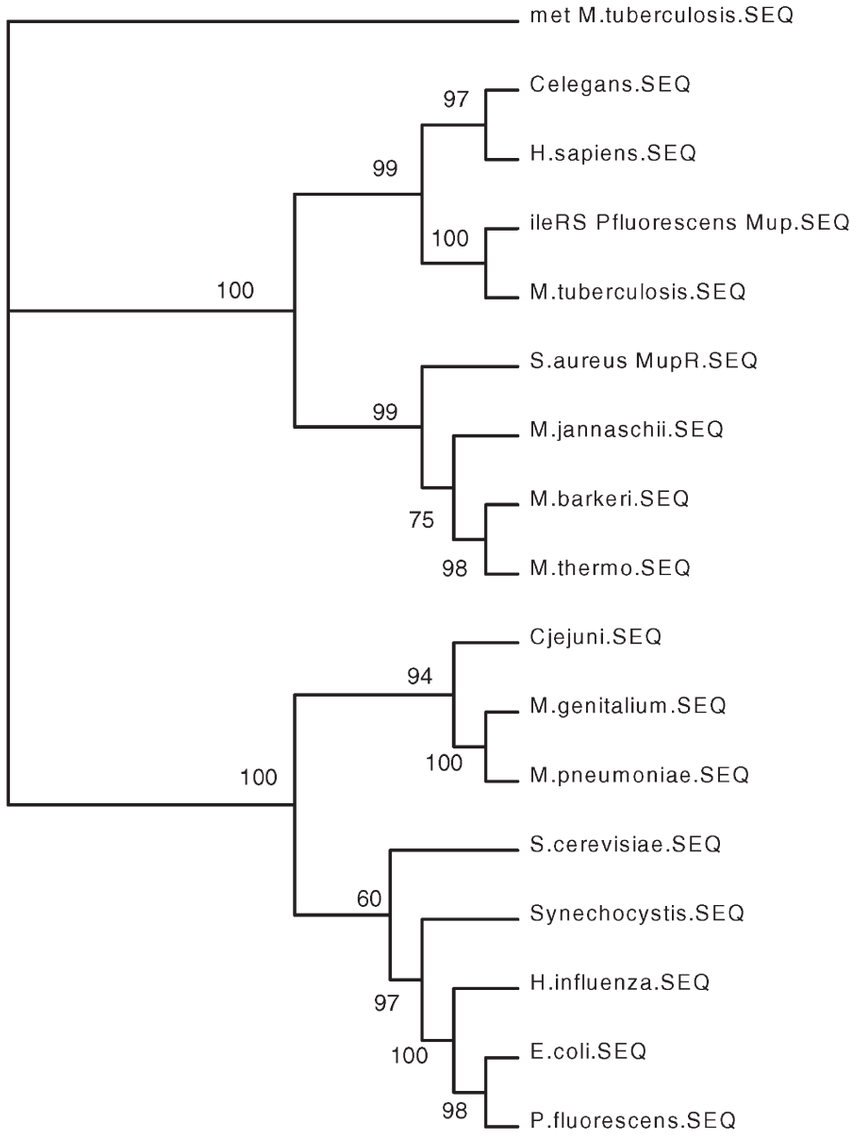

FIGURE 4 Inferred phylogeny of the *P. flourescens* rILERS protein, based on maximum parsimony analysis of nucleotide data with 1000 bootstrap replications and 50% consensus rule in effect. The percent bootstrap support is indicated at each node. The *metRS*_gene of *Mycobacterium tuberculosis* was used as an outgroup.

from *P. fluorescens* 10586 is more closely related to MupA (56% similarity) than the ILERS previously identified in this strain of *P. fluorescens* (26% similarity).

Brown *et al.* (1998) suggest that the mupirocin-resistant forms of ILERS in prokaryotes originated from eukaryotes. Although this is a plausible hypothesis, it is important to note that the *rILERS* gene described in this study originated from a mupirocin-producing strain of *P. fluorescens*, and this gene has not been previously described. *P. fluorescens* is a common inhabitant of the soil and rhizosphere where competition between microbes is intense. Mupirocin production by *P. fluorescens* may have provided this bacterium with a competitive advantage that was eventually reduced with the transfer of *rILERS* to other soil-dwelling prokaryotes. This would help explain why mupirocin resistance was present long before the clinical use of the antibiotic (Rahman *et al.*, 1990). Therefore, it is possible that this gene was horizontally transferred from *P. fluorescens* to gram-positive organisms such as *S. aureus* and *M. tuberculosis*, which have eukaryotic hosts.

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